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## MINING THE PULSE SEED COAT FOR DIETARY BENEFITS AND ECONOMIC ADVANTAGES

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# MINING THE SEED COAT FOR DIETARY BENEFITS AND ECONOMIC ADVANTAGES

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**Final Report**

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**Investigators:**
Susan Marles[1] and Kirstin Bett[1]

**Collaborators:**
Tom Warkentin[2], Bert Vandenburg[2] and Ed Krol[3]

[1]Department of Plant Sciences
[2]Crop Development Centre
[3]Department of Pharmacy & Nutrition
University of Saskatchewan

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## A. ABSTRACT

The seed coat of genotypes of dry bean (black, red, pinto), field pea (yellow, green, maple and dun) and faba bean were examined for bioproducts related to the functional food character. All seed coat or hull tissues contained flavonoids (dry bean, faba bean) or carotenoids (field pea). The pigmented seed of maple and dun types of field pea also contained condensed tannin, a flavonoid product. Flavonoids in dry bean were represented mainly by two flavonols (kaempferol and quercetin), condensed tannin and trace amounts of (+)-catechin / (-)-epicatechin. In black bean, significant differences related to genotype rather than environment, indicated by the low variability amongst plants from different plots within each genotype. The G x E interaction was not significantly different for anthocyanin ($p$ = 0.4548), but was significant for extractable condensed tannin ($p$ = 0.0708) and highly significant for the bound anthocyanidin trait ($p$ = 0.0050). Polyphenol oxidase activity, responsible for postharvest darkening of seed coat tissue, may ameliorate some of the available functional attributes by converting the flavonoids to quinones. In field pea, carotenoids were predominantly present as lutein, chlorophylls *a* and *b*, and traces of zeaxanthin. In developing hull material of field pea, other carotenoid metabolites (violaxanthin, antheraxanthin, neoxanthin, and β-carotene) were accumulated although these largely disappeared by maturity. Genotype was significant in affecting the phenotypic accumulation of lutein and chlorophyll *b* over both year and location ($p$ = 0.016, 0.018, respectively). The G x E interaction was significant for chlorophyll *a* ($p$ = 0.002), but was not significant for the other compounds ($p$ = 0.296) taken over all genotypes. Edible pulses consumed as a whole food can contribute to good health due to the total functional food profile. The hull tissue derived from dehulling has the potential to enrich other foods lacking in fibre and dietary amendments that contribute to the health benefits of the foregoing bioproducts.

## B. EXECUTIVE SUMMARY

Pulses (edible legumes) are known to be healthy for many reasons and as such have been part of the human diet for centuries. With no knowledge of biochemistry, ancient civilizations realized that a healthy populace required a cereal and a legume in the diet. With the exception of dry bean, the seed coat is often discarded and considered to have little value. In the case of bean, the seed coat colour is very important as a visual trait and the colour dictates what market it is sold into. Compounds that are associated with pigment along with some colourless compounds found in these seed coats, however, are also of nutritional benefit. Many such as carotenoids and polyphenolics reportedly have anti-oxidant

properties and antiproliferative effects, anti-inflammatory changes and benefits positively influencing atherosclerosis have also been claimed. Whole grains rich in these compounds can be considered "functional foods" and should be part of a healthy diet. Incorporation of hulls (seed coats), currently a by-product, into food items would not only result in increased fibre but potentially other beneficial compounds. The interest in functional foods has resulted in a number of new food products in the marketplace designed to address specific health concerns, particularly with regard to chronic diseases associated with aging.

Simply based on pigment variation we know that there is variability for at least some of these compounds in the seed coats of pulse crops. Biochemical analyses allow us to determine which compounds are present and how much variability there is among genotypes. Identification of the presence of genetic variability would allow breeders to target specific compounds for increase (or decrease) to develop new cultivars with superior nutritional characteristics. The development of biochemical tests suited to breeding programs (i.e. reasonably quick and inexpensive) is necessary if this is to become a target for selection. Proper understanding of the biochemistry of the material we work with will also allow us to develop populations designed to identify molecular markers for these compounds which will greatly facilitate the selection process.

In this project we looked at the seed coats of dry bean (pinto, black and red), field pea (green, yellow, dun and maple) and faba bean. We did not look at lentil as this is being carried out under a different project. We found variability in polyphenolics, chlorophylls and carotenoids which suggests it would be possible to breed for different levels of these types of compounds. Knowing the biochemistry of the seed coat has given us some insight into why some genotypes are slightly different than others. For example, CDC Rio has not become an accepted black bean cultivar because its seed coat colour can be variable and is not black enough. This is likely because it is much lower in anthocyanins that the other black beans. In field pea, the level of lutein is higher in the dun and maple pea types examined than in the yellow or green types. Incorporation of less than 1 gram of hull from dun or maple peas could be sufficient to meet the RDA for carotenoids. It would be interesting to see if increasing lutein is possible in green or yellow pea hulls without compromising the quality of cotyledons or the visual appeal of the whole seed.

This is important knowledge with the potential for an economic advantage for Saskatchewan growers. The accumulation of a range of health-giving bioproducts, such as flavonoids and carotenoids, in the pulse seed coat provides a splendid potential for an unrealized source of dietary components. These classes of compounds have been reported as important dietary factors using in vitro techniques,

but follow-up clinical studies are required to make this a reality. To develop healthful pulse products, it is critical to understand the nature of the pigment in the seed coat, and what other health-giving bioproducts occur as colourless components. Very little is known about the accumulation of these added-value dietary prospects. The emerging biochemical profiles of beans and peas could be the means of developing a reputation for Saskatchewan as a promising supplier of highly flexible pulse crops with regard to different utilization strategies.

## C. TECHNICAL REPORT

### C.1. INTRODUCTION

This project was designed to improve our knowledge of naturally-occurring bioactive products (functional food characteristics) in several pulse crops (edible legumes) adapted for western Canada. The philosophy of this project asserts that the functional food characteristic of pulse crops would be furthered if a distinct biochemical character (a 'chemotype') could be followed during variety selection trials. Identification of compounds with reported health-related attributes complements the marketing strategies of appearance, superior nutrition and cooking-traits that are currently used to promote pulse crop consumption. The chemotyping profiles so generated would validate the inclusion of Saskatchewan pulses in the functional food category. With suitable clinical trials, Saskatchewan pulses may then be promoted as having important dietary factors that are documented to contribute to managing diseases such as colon cancer, diabetes, cardiovascular disease and atherosclerosis (*1-11*).

Cultivars of dry bean (black bean, small red and pinto) (*Phaseolus vulgaris* L.), and field pea (yellow, green, dun and maple types) (*Pisum sativum* L.) provided the material that was characterized. In addition, some faba bean (*Vicia faba* L.) breeding lines were assessed. Lentil cultivars were not characterized in this project as this work is being carried out under B. Vandenberg's NSERC-IRC program. Biochemical assays appropriate for surveying the functional food chemotype profile in the seed coat across biological replicates and growing locations were developed for these classes of pulses. These screening protocols facilitated identification of functional food attributes under typical growing conditions in prairie envionments. It became evident early on in the project that we needed to look at multiple reps across multiple locations to ensure that the amounts of these compounds that we were finding were due to genotype rather than environment so many more samples were assayed than originally intended. In this report, we show that genotype generally dictates the chemotype and that

most functional food bioproducts reliably accumulated in the selected genotypes irrespective of the growing environment.

### *C 1.1 The functional food attribute*

Recommendations for healthy diets point toward consumption of pulses because these crops are rich in desirable functional food bioproducts such as carotenoids, polyphenolics and other compounds with the capacity for these effects (*4, 7, 8, 11-15*). The holistic term 'functional food profile' frequently includes the nutraceutical category as well which is defined as "...any substance that is a food, or part of a food, and provides medical or health wellness benefits, including the prevention or treatment of disease" (*16*). However, the notion of a "functional food attribute" is a concept rather than a universally-accepted definition. In the context of our research with pulse crops, it may be more appropriate to define this attribute as a food product that can be regarded as 'functional' if the product benefits one or more physiological functions in the body, coincidentally ensuring an amelioration of the risk of disease beyond satisfactory nutritional effects (*6*). An important consideration when speaking of "functional food" is that it must remain as food, and deliver the health benefits in amounts that can normally be expected to be consumed in the diet as part of a normal food consumption pattern, rather than taken as a pill, capsule or tincture (*17, 18*).

### *C 1.2 The role of polyphenolics in the functional food profile.*

Polyphenolics, especially flavonoids such as condensed tannins (*syn.* proanthocyanidin, CT) catechins, anthocyanins, isoflavones and flavonols (**Figure F. 2.1**), are considered important functional food characters connected with a good health and wellness profile in nutrition. Frequently, the antioxidant effect of polyphenols, together with the antiproliferative effects of flavonoids, are reported to contribute to wellness based on their documented influence on urinary tract infections (*19*), induced apoptosis for controlling cancer cell proliferation (*20*) and similar other physiological phenomena (*2*). These and other reports support the recommendation by national public health agencies of a diet with increased consumption of flavonoid-rich food such as fruit and the edible legumes (*13, 21*). It has been demonstrated that potentially valuable compounds such as the phenylpropanoid and flavonoid families of chemicals (ferulic acid and related hydroxycinnamates, quercetin, kaempferol, catechin, epicatechin, dihydroflavonols and condensed tannins, as well as various polymeric associations of these polyphenolics) accumulate in the seed coat (*22, 23*). However, polyphenolic differences are known to be a malleable reflection of phenylpropanoid or flavonoid metabolism in a given growing season. For

functional food attributes as well as consistency in appearance, it is important to determine whether flavonoid differences are a reliably expressed trait by the genotype or randomly associated with the visual phenotype and dependent on growing conditions.

The occurrence of unoxidized polyphenolic metabolites (*e.g.* flavonols) is visually undetectable at harvest because these compounds are colourless, mainly tasteless components. Often, the seed coat darkens after harvest because this tissue harbours an invisible quantity of these unoxidized chemicals. Like other harvested foods such as root vegetables, the oxidation products become evident only during storage since they are prone to conversion to quinones by polyphenol oxidase enzymes (PPO) or catabolized to hydroxybenzoates (*24-26*). Quinones are very unstable products which react quickly either by polymerizing with proteins or undergoing auto-oxidation reactions to become visibly brown, red-brown or black pigments. Often these polymers render the converted metabolites unextractable and create an unattractive change in appearance. In cereals, processing activates PPO activity and causes discoloured noodle products (*27, 28*); thus, post-harvest changes in pulses during storage are not unexpected to be associated with quinone formation, catabolism of phenolics and auto-oxidation of the polyphenolic-rich seed coat.

In addition to cataloguing the occurrence of the intermediary products of flavonoid biosynthesis, CT accumulation is important to monitor. Considerable research has been devoted to putative anti-nutritional effects that CT can have on the diet (*29-31*). However, these studies did not consider the effects of the CT catabolites deriving from human metabolism and may have been unduly influenced by the results of livestock nutritional research (*32-35*). *In vitro* studies have shown that CT can be transformed into phenolic acids that have a beneficial effect on the gut and kidney functions (*36*). It so happens that the bulk of nutritional research is inconclusive on the value of CT in the human diet. From the evidence of flourishing health in cultures where edible pulses form a major part of the diet, it would be unreasonable to exclude legumes with a CT-rich seed coat from the healthy functional food category.

In the research reported here, the occurrence of polyphenolics in a range of pulse crops was documented with the intention of providing plant breeders with a catalogue of genotypes and associated putative dietary benefits as well as an indication of the capacity for quinone-related post-harvest colour changes. In this way, cultivar development in pulse crops can target both functional food traits and eliminate the potential for undesirable changes in post-harvest appearance. These objectives lead western Canadian pulse crop development towards enhancement of desirable dietary profiles and stable

visual phenotypes. Consequently, the data generated in this project can deliver information for product advantages for Saskatchewan pulse growers.

### *C. 1.3 The role of carotenoids in the functional food profile*

In the original research proposal, carotenoids and associated products such as chlorophyll were not intended for investigation. However, it turned out that the field pea genotypes (with two exceptions, dun and maple types) were rich in these bioactive products rather than polyphenolics and warranted detailed analyses. Carotenoids and chlorophylls are long chain, unsaturated hydrocarbons, derived from a product of terpenoid metabolism (**Figure F. 2.2**), and form critical nutritional precursors in human nutrition (*37, 38*). Carotenoids include the polar, oxygen-containing xanthophylls and the lipophilic carotenes, which are both responsible for controlling reactive oxygen species by their antioxidant activity (*2*). Chlorophylls, with their well-documented role in photosynthesis, also absorb light-mediated oxygen radicals and mediate the effects of these reactive molecules on membranes (*39*). Consumption of carotenoids has proven beneficial when taken in recommended daily amounts (RDA) to obtain retinol (Vitamin A) and to ameliorate reactive oxygen species (*14, 40, 41*). Hence, these long-chain molecules are important natural plant products to consider when characterizing pulses for their functional food attributes.

## C. 2 OBJECTIVES

The research project featured three key objectives in chemotyping pulse crops grown on the western Canadian prairies:

### *C. 2.1 Enhance marketing advantages*

The pulse export market is attracted to elite products that target appearance and nutritional advantages. The market value can therefore be increased for the cultivars with documented health-giving benefits coupled with superior visual appeal that remains stable during a reasonable storage term. To this end, the project's practical objective was to screen agronomically-superior pulse cultivars for the occurrence of polyphenolics and other compounds related to functional food criteria in a range of pulse crops and to evaluate the variabilty of these compounds by assessing different genotypes within each market class.

### *C. 2.2 Crop utilization innovation for value-added opportunities*

Waste-products such as the hulls (*e.g.* from field pea splitting) can have value as a food additive. Chemical components that are attractive for antioxidation effects in processed food or for other nutritional attributes will be identified and provide added-value by using plant byproducts that currently are discarded. Other opportunities for crop utilization will be monitored where chemotyping deomonstrates this may be possible.

### *C. 2.3 Catalogue of genotypes and relevant bioproduct capacity*

The final objective was to develop a functional foods catalogue of pulses based on their seed coat character that reflects the biochemical profile. The format will be a searchable chemotype database of the bioproducts and related phenotypic information. The variabilty of the trait, determined by assessing assorted genotypes within different market classes, will reflect the reliability of the chemotype. The design of this databank could provide the basis of new plant breeding objectives in the development of pulses for premium markets. In addition, the chemotype catalogue encourages research and development in pulses and will be augmented by publishing journal articles and giving conference presentations pertaining to our pulse research discoveries.

## D. RESULTS BY MARKET CLASS

### *D. 1. Black Bean*

Black bean cultivars were developed for their uniformly black seed coat appearance. The most attractive colour in the archetypical genotype has no overtone of brown and desirable cultivars produce consistent "black" phenotypes despite environmental stresses. The black colour is actually a deep purple mix of anthocycanins (glycosylated flavonoids), with overtones in the violet or blue end of the spectrum. The classic phenotype is typically rich in delphinidin with supplementary amounts of other anthocyanins (*42, 43*). In past research with older varieties, selected Canadian black bean cultivars were assessed for anthocyanins in whole, milled bean (*44*) and for overall canning quality (*45*). Although black bean anthocyanins have been widely described (*43, 46-48*), a comprehensive genotype by environment (G x E) assessment of the effect of growing location on the overall polyphenolic characteristics and concentration of individual classes of flavonoids has not been reported, particularly from the Canadian short season growing areas. Thus, flavonoids in seed coat tissue were quantified from four black bean genotypes grown at four locations, with the objective of assessing the polyphenolic variation and G x E effects. The genotypes (UI 911 [UI]; CDC Rio [Rio], AC Black Violet [BV], and CDC Jet [Jet]), were

grown in a replicated random complete block design and harvested in 2006 from four locations, (Carman, [C], Portage [P], Morden [M], and Winkler [W], MB). Three biological replicates of each genotype were obtained from the Winkler location, for polyphenolic tests of variability within one environment. To assess the G x E effect, composite (bulked) samples were taken from each location (C, P, M, W) to provide replicate subsamples of each genotype.

***D. 1.1 Overview of key results from black bean investigations***

*D. 1.1.1. Methods development.* A rapid mini-assay protocol for screening pulse crop accessions was developed to extract and quantify abundant polyphenolics in the seed coat. This procedure employed the novel approach of three sequential extraction procedures to quantify three forms of polyphenolic seed coat pigments from one tissue sample. Sequential extracts consisted of ***1***, 80% acidified methanol, ***2***, 70% acetone, ***3***, *n*-butanol-HCl. The miniaturized extraction protocol was tested for efficiency and found to be reproducible for seed coat weights between 15 to 70 mg. By changing from multiple methanol-extractions with sample concentration procedures, customary in long-established practices (*43*), to a 4-h extraction using one 80% aqueous methanol acidified with 0.05% trifluoracetic acid, the large-scale genotype screening assays resulted in a precise and reproducible quantification of anthocyanins and could detect other polyphenolics such as flavonols and the flavan-3-ols. The subsequent extraction with 70% acetone was used to quantify the soluble CT and was followed by heating of the pellet in *n*-butanol-HCl to depolymerize the bound CT for quantification by the method established by Terrill *et al.* (1992) (*49*). The rapid miniaturized protocol is described in detail in the journal article ensuing from the black bean research (*42*). This protocol must always be preceded by larger-scale extractions for each genotype to ascertain which bioproducts are present. The lower tissue weight in the mini-preparations can then be adjusted to suit the metabolic profile, should trace amounts detectable in larger preparations be otherwise undetectable.

*D. 1.1.2. Chemotype characterization of black bean genotypes.* Quantification by HPLC revealed that one black bean genotype (CDC Rio) accumulated less anthocyanin than the other genotypes examined (**Figure D. 1.1**), although this lower amount in CDC Rio was masked by the co-occurrence of CT (**Figure D. 1.2**). The anthocyanin elution pattern was consistent over all extractions within each genotype in the elution order of delphinidin, petunidin and malvidin. Malvidin was the most likely to become undetectable in the preparations because it was highly labile unless the extracts were analysed promptly. Occurrence of a fourth anthocyanin moiety was evident in CDC Jet (arrow, **Figure D. 1.1**), similar to that reported in other accounts of black bean seed coat analyses (*43, 50*). The largest

concentration of pigment belonged to the bound anthocyanidin category (**Figure D. 1.2**). Extractable anthocyanin had the lowest concentration, reflected in the visible differences reported in the following section on histochemistry (D 1.1.3). Statistically significant differences related to genotype rather than environment, signified by the low variability amongst plants from different plots within each genotype (**Table D. 1.1**). The G x E interaction was not significantly different for anthocyanin ($p = 0.4548$), but was significant for extractable CT ($p = 0.0708$) and highly significant for the bound anthocyanidin trait ($p = 0.0050$). Refer to **Appendix 9.2** for statistical procedures.

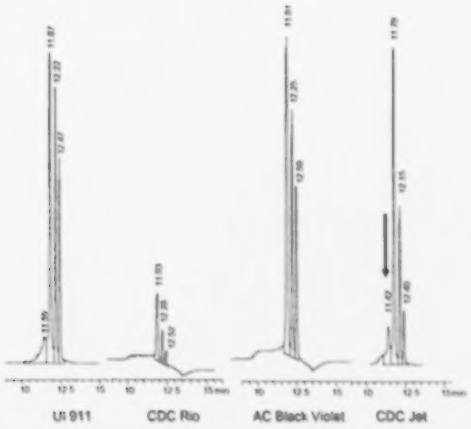



**Figure D. 1.1 Elution profiles exposed inherent differences in four genotypes of black bean.** Extract volumes were adjusted to show relative concentrations of anthocyanins (mg $g^{-1}$ seed coat), using the unhydrolysed rapid, mini-extraction procedure. Separations were obtained by HPLC. Anthocyanin elution patterns were very consistent among genotypes and the wavelength maximum (530 nm) used to monitor the different compounds by UV diode array was reliable in detecting anthocyanin. Arrow indicates an unidentified anthocyanin.

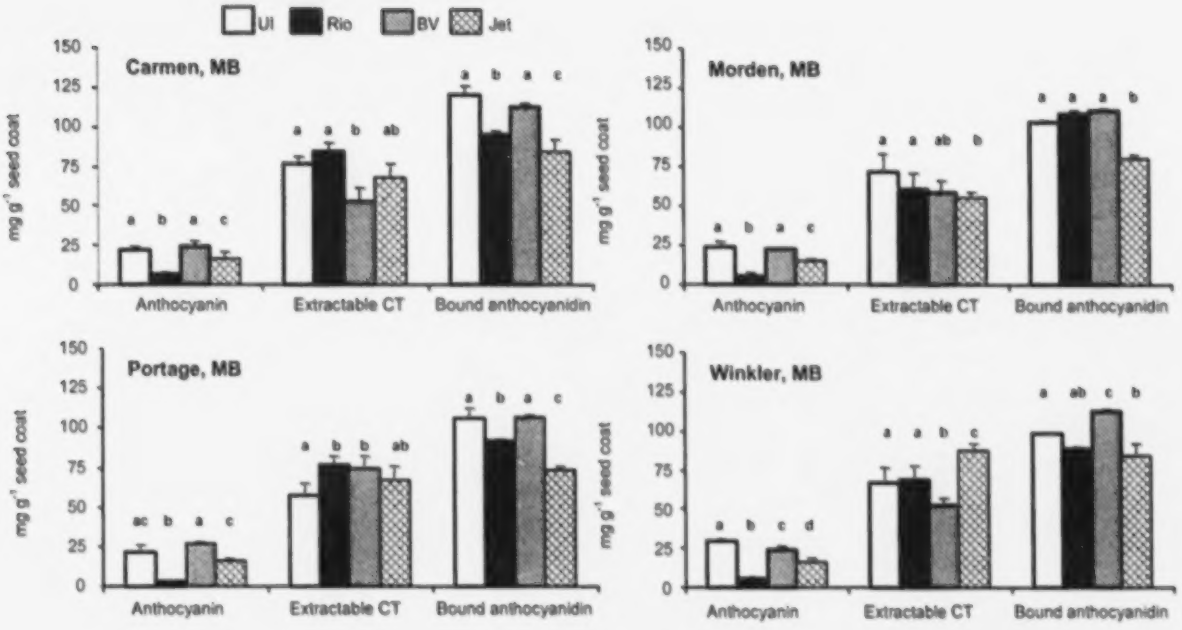



**Figure D. 1.2. Flavonoid attributes of black bean genotypes grown at four different locations.** Graphs are presented by location for each flavonoid trait. Within each flavonoid category, means with the same letter are not significantly different at the indicated location. *Assays:* anthocyanin and extractable CT (analysed by UV-spectral detection) and bound anthocyanidins of otherwise unextractable CT/anthocyanin pigments (using the method of Terrill et al., 1992) (*41*). The assays contained three composite replicates from each location per genotype and were repeated at least three times. The results are reported as means of each genotype at each location; the standard error bars represent ± standard error of the means. *Genotype codes:* **UI**, UI 911; **Rio**, CDC Rio, **BV**, AC Black Violet; **Jet**, CDC Jet.

**Table D. 1.1: Analyses of type 3 tests of fixed effects* among black bean genotypes from four locations (harvested 2006, shown in Figure D. 1.2).**

| Effect | F Value | $P > F$ |
|---|---|---|
| | *Anthocyanin* | |
| Genotype | 63.79 | < 0.0001** |
| Location | 0.72 | 0.5532 NS |
| Genotype*location | 1.04 | 0.4548 NS |
| | *Extractable CT* | |
| Genotype | 2.05 | 0.1269 NS |
| Location | 1.01 | 0.4007 NS |
| Genotype*location | 2.01 | 0.0708* |
| | *Bound anthocyanidin* | |
| Genotype | 49.75 | < 0.0001** |
| Location | 4.78 | 0.0054** |
| Genotype*location | 3.12 | 0.0050** |

*Generally-applied statistical details, refer to Appendix 9.2

[c] Contrast statements in Proc Mixed (SAS) were used to compare genotypes and labels appear on Figure D. 1.2 to indicate which genotypes significantly differ.

NS, not significant; * significantly different (90%); **highly significantly different (95%)

*D. 1.1.3 Differential accumulation of flavonoid products in seed coat integument layers.* Black bean belongs to a bean group that is characterized, in addition to other traits, by having a seed coat that readily separates into inner and outer integument layers (*51, 52*). Pinto bean genotypes do not do so, and the CT appears to occur entirely in parenchyma cells only, at the outermost cells next to the palisade layer. In the black bean genotypes, the outer integument turned bright, cherry red in diagnostic histochemicals within 30 s whereas the inner integument, with the visible pigments in isolated patches, reacted slowly and did not turn red until after several minutes (**Figure D. 1.3**). According to results in other species, this was typical for the differentiation between anthocyanins (in the outer integument) and CT (in the inner integument)(*53*).

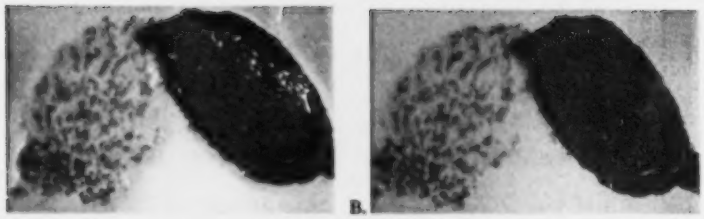


**Figure D. 1.3.** Histochemical reaction of pigments in dissected integuments of black bean. Integument layers of CDC Jet **(A)** before staining in *n*-butanol-HCl, inner integument (left) and outer integument (right); **(B)** after staining in *n*-butanol-HCl (magnification: 3.2 x). Other black bean genotypes produced the same result.

### *D. 1.2 Future perspectives in black bean functional food attributes.*

Potential unfavourable quality attributes, in the form of high concentrations of CT, were evaluated in decorticated seed coat tissue, in conjunction with the occurrence of other polyphenolics such as anthocyanins and trace flavonoid metabolites. Tissue-specific expression of CT and anthocyanin in the seed coat integuments is reflective of complex metabolic partitioning in black bean. The anatomy of readily-separated integument layers accumulating characteristic bioproducts may be part of an intricate metabolic partitioning cascade uniquely associated with black bean genotypes. Such differentiation between seed coat layers may lend itself to cultivar development, because consideration of such tissue targeting may become important should plant breeders want to address problems such as the ones evident in the pigmentation of CDC Rio. The lack of a uniform purple-black appearance and a distinct brown tone is reflective of the CT-rich phenotype that this cultivar revealed in the bound and extractable CT analyses. These results may also provide plant breeders with an insight into the genotypic and environmental effects on visual phenotypes having beneficial dietary traits. Accordingly, black bean cultivars could be developed to target known functional food traits and enhance desirable dietary profiles, while avoiding traits such as insufficient anthocyanin accumulation that detracts from appearance.

### *D. 2 Small red bean*

Small red bean is distinct from the larger red kidney market class, the latter not being suited to the short growing season in the Canadian prairies. Several genotypes have been developed, mainly in Alberta and Manitoba, Canada and the cultivars emphasize upright growth, yield, disease resistance and early maturity (*54-56*). At present, red bean cultivation is dominated by AC Redbond due to its ease of harvesting and superior yield (P. Balasubramanian, 2009, pers. comm.). However, as with other dry

bean markets, visual appearance is important and AC Redbond was somewhat darker than AC Earlired and AC Scarlet. Marketing of AC Redbond must be done soon after harvest to avoid downgrading due to darkening of the seed coat. In the research here, a breeding line, L98D347a, a sibling of lines that lead to development of AC Redbond and AC Earlired, was added to the foregoing genotypes to investigate the abundance and variability of flavonoids as part of this functional food attribute survey (**Table D. 2.1**). All samples were provided as biological replicates as either plot composites in replicate or as individual plot replicates for each location. Refer to **Appendix 9.2** for statistical procedures.

**Table D. 2.1.** Record of small red bean genotypes and harvest data

| Genotype | Year harvested | Location |
|---|---|---|
| AC Scarlet | 2006 | Morden, Carman (MB) |
| | 2007, 2008 | Morden, Carman, Winkler, Portage (MB) |
| AC Earlired | 2004 | Vauxhall (AB) |
| | 2009 | Lethbridge, Vauxhall, AB |
| AC Redbond | 2004, 2007 | Vauxhall (AB) |
| | 2009 | Lethbridge, Vauxhall (AB) |
| L98D347a | 2007 | Vauxhall (AB) |

### *D. 2.1 Overview of key results from small red bean investigations*

Unlike black bean, red bean does not accumulate anthocyanin compounds in the seed coat. The red colour of the seed coat is derived from conversion of colourless condensed tannin units, flavonols and flavan-3-ols such as catechin, to produce cross-linked or oxidized molecules (quinones, hydroxybenzoates) that can occur in a range of red to brown colours. Such changes have been observed for these metabolites in other seed coat pigments (*57*).

*D. 2.1.1 Chemotype characterization of small red bean genotypes.* Variability in terms of CT concentration was found in red bean, namely that genotype had a significant effect on the accumulation of CT (**Figure D. 2.1**). The location effect on red bean CT accumulation was less evident than environmental effects by year. This was particularly noticeable when comparing the CT concentration of AC Scarlet in 2007 from four locations in Manitoba with the CT concentration in this genotype in 2008 grown at the same four locations (**Figure D. 2.1**). However, AC Redbond remained the genotype that accumulated the most CT and there were no crossover interactions with other years and genotypes. Both year and location appeared to affect kaempferol accumulation (**Figure D. 2.2**). AC Redbond

contained greater amounts of kaempferol than other cultivars, although the L98D347a line was very similar. One cultivar, AC Scarlet, contained only trace amounts of kaempferol, compared to the other cultivars (arrows, **Figure D. 2.2**). An experimental preparation of AC Scarlet using an order of magnitude more seed coat tissue as the starting point in the extraction did yield detectable kaempferol in the HPLC separation. Thus it is evident that the flavonols synthase pathway is not entirely turned off in this genotype and perhaps controlled by an environmentally-responsive promoter.

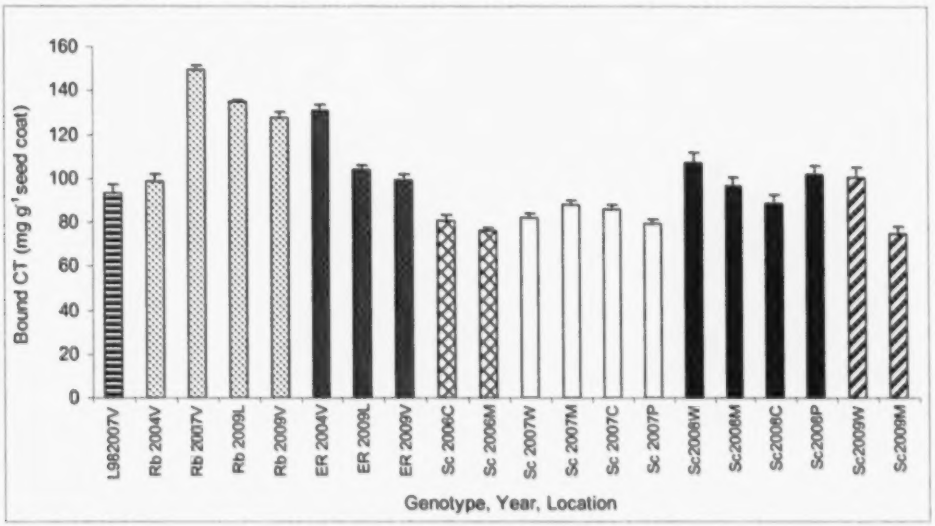



**Figure D. 2.1 Concentration of bound condensed tannin (CT) in genotypes of red bean from different locations and years.**

CT was quantified by butanol-HCl assays *(41)* in several locations over different years. Genotype was the significant factor in differences. SE bars represent the standard error of the means of biological replicates (n=3 for years before 2007; n=4 for 2007 to 2009). Locations in Manitoba (***C, M, W, P***) are composite technical replicates bulked from each location..

*Figure legend:* genotype code is followed by the year grown and a single letter to designate the location. *Genotype:* ***L98***, germplasm line L98D374a; ***Rb***, AC Redbond; ***ER***, AC Earlired; ***Sc***, AC Scarlet. *Location:* ***V***, Vauxhall, AB; ***L***, Lethbridge, AB; ***C, M, W, P***, Carman, Morden, Winkler, Portage, MB (respectively).

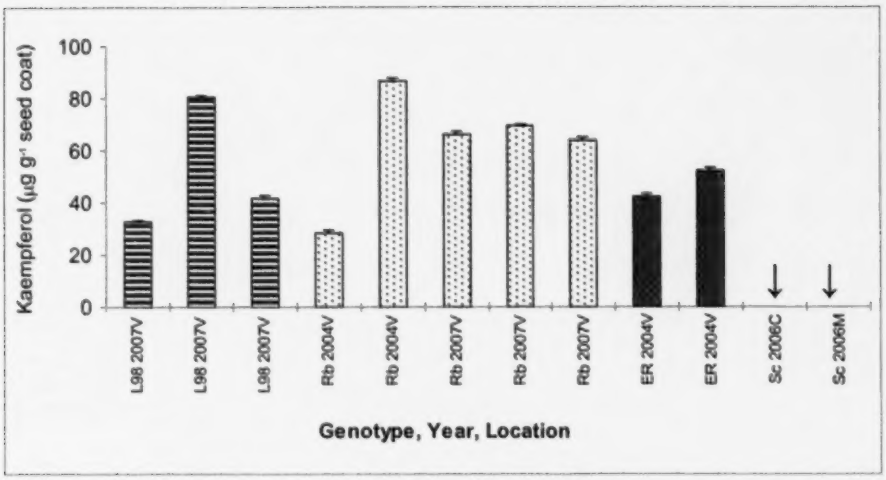



**Figure D. 2.2. Concentration of kaempferol in red bean genotypes grown in different environments and over three years.**

Kaempferol was quantified by separation and UV-detection on the HPLC *(25)* in several locations over different years. Genotype was the significant factor in differences. AC Scarlet contained no detectable kaaempferol (arrows). SE bars represent the standard error of the means (n=3) of technical replicates from each biological (plot) replicate. Locations in Manitoba (***C, M***) are composite replicates bulked from each location.

*Figure legend:* genotype code is followed by the year grown and a single letter to designate the location. *Genotype:* ***L98***, germplasm line L98D374a; ***Rb***, AC Redbond; ***ER***, AC Earlired; ***Sc***, AC Scarlet. *Location:* ***V***, Vauxhall, AB; ***C, M,*** Carman, Morden, MB (respectively).

### *D. 2.1.2 Future perspectives in small red bean functional food attributes.*

As discussed in a previous section on functional food attributes of flavonoids (**C. 1.2**), kaempferol and CT are amongst the most desirable of secondary metabolites to enhance the health-giving character of edible pulses. This leads to some difficulties in promoting AC Scarlet with its remarkably lower kaempferol and CT content. However, appearance is an important factor in red bean marketing so the genotype is a valuable resource for its apparent differences in flavonoid products. It was also observed in the large scale preparation (**D. 2.1.1**) that traces of quercetin were present. This finding led to a recent testing of a few other genotypes with larger scale preparations and it was noted that quercetin occurred in all the genotypes examined. For this reason, an addendum to the red bean flavonoid profile will be reported at a later date so that a more accurate functional food profile for the red bean genotypes will be available. At that time, a larger number of populations from the last growing season will improve the data available to correctly assign statistical values and classify which are the significant differences.

Since the difference in kaempferol concentration was implicated as one of the significant differences between populations of regular- and slow-darkening pinto bean lines (*58*), this flavonoid is important to document further in developing red bean genotypes from the post-harvest darkening standpoint. Flavonols may contribute to darker seed coat colour more readily than CT by virtue of their ability to oxidize more quickly and initiate ortho-quinone formation (*59-61*). This rapid post-harvest change is an important contribution to darkening and the near-absence of kaempferol in AC Scarlet compared to the other genotypes may lead to some plant breeding initiatives to enhance small red bean colour. However, the enzyme chiefly responsible for colour changes related to quinones was not assayed in red bean for the reason that darkening has not been identified as a major issue in marketing the crop. Overall, the detailed survey of small red bean provided a broad picture of the environnmental effects on the accumulation of pigment and other polyphenolic compounds in the seed coat.

### *D. 3 Field Pea*

Functional food attributes of field pea, particularly of the seed coat (hull) have not been reported previously in the literature. Notably, the hull, has attracted very little attention as a nutritional source, the main focus being whether its appearance is pigmented or translucent (unpigmented). The immediate objective in the research was to determine which (if any) secondary metabolites with functional attributes might predominate in the field pea hulls. In addition, there was interest from the plant breeding perspective in whether metabolites specific to the flavonoid (**Figure F. 2.1**) or carotenoid (**Figure F. 2.2)** pathways were uniquely associated with distinct classes of field pea (yellow, green, dun or maple types). An important corresponding objective was to discover whether the plant natural products accumulated differently, relative to environmental effects, amongst genotypes. To this end, mature field pea samples harvested in 2007and 2008 from two locations (Rosthern [Ros] and Sutherland [Suth], SK) were evaluated to examine how uniformly the bioproducts accumulated over time and location amongst the genotypes.

Secondly, the hull plays an undetermined role in preventing cotyledon bleaching in some green cotyledon types (*62*). Phenotyping for this trait would be furthered if a distinct chemotype linked to bleaching resistance was elucidated. Constraints in selecting for the bleaching resistance trait are imposed by difficult genetic characterization (*62*) and also by environmental effects that confound selection for resistance by genotype alone (*63, 64*). Thus, a secondary objective to profiling functional

food characteristics in field pea hulls included evaluating differences in compound classes in the field pea hull tissue of some genotypes known to have reduced or no bleaching resistance (**Table D. 3.1**). For these reasons, measurement of specific bioproduct accumulation in developing hull tissue of yellow, green cultivars (both bleaching resistant green types [R] and not resistant [NR] to bleaching) was followed over a period of 7 to 28 days after flowering (daf). This appraisal was expected to monitor phytochemical changes to the metabolic profile during seed filling. Taken together, these data may provide an insight into whether there are genotypic advantages with respect to desirable functional food attributes, including chemotypes that may contribute to bleaching resistance.

**Table D. 3.1 Genotypes used in profiling bioproducts in field pea hulls.**

| *Genotype*[1] | *Category* | *Phenotype (hull / cotyledon colour)* |
|---|---|---|
| CDC Rocket[2] | maple | brown (dark pigmented / yellow) |
| CDC Dundurn[2] | dun | tan (pale pigmented / yellow) |
| Cutlass[2, 7] | yellow | translucent (unpigmented / yellow) |
| CDC Prosper[2] | yellow | translucent (unpigmented / yellow) |
| Eclipse[3] | yellow | translucent (unpigmented / yellow) |
| Agassiz[4] | yellow | translucent (unpigmented / yellow) |
| CDC Tucker[2] | yellow | translucent (unpigmented / yellow) |
| Orb[5, 7] | green | translucent (unpigmented / green) (NR) |
| CDC Striker[2, 7] | green | translucent (unpigmented / green) (R) |
| CDC Patrick[2] | green | translucent (unpigmented / green) (R) |
| Cooper[3] | green | translucent (unpigmented / green) (NR) |
| SW Sergeant[6] | green | translucent (unpigmented / green) (R) |

[1]Cultivar and affiliation; [2]CDC, Crop Development Centre, University of Saskatchewan, Saskatoon, Canada; [3]Cebeco Zaden, The Netherlands; [4]Agriculture and Agri-Food Canada.; [5]Sharpes, UK; [6]Svalof Weibull AB, Sweden; [7]Genotype also grown in the greenhouse for developing tissue samples, 2009. NR, not resistant to bleaching; R, resistant.

### *D. 3.1 Overview of key results from field pea investigations*

*D. 3.1.1. Methods development: tissue collection and extraction.* An average yield of 9.8% (± 0.5%) hull tissue from the original seed sample (weighed before and after dehulling and fractionation) was obtained from mature, whole seed (Sataki Dehulling Mill) followed by air fractionation to remove cotyledons remaining after dehulling. At least 1 g mature hull tissue was required to produce UV-detectable carotenoids in concentrated extracts. In developing tissue, concentrated extracts of at least 0.2 g tissue was required to detect the predominant carotenoids. Extraction efficiency is an important

parameter in order to accurately reflect the real differences among genotypes. A wide variety of solvents has been reported for the extraction of chlorophyll and carotenoids from a range of plant species and other sources (*62-69*). Similar to published records, it was established that chlorophyll in field pea hull was soluble in a greater range of solvents than were the carotenoids (**Table D. 3.2**).

In the results reported here for each chemical, the chlorophylls, xanthophylls and carotenoids were found to be readily solublized using the MeOH/DCM (1:1) solvent, similar to that reported for durum wheat (*70*). In addition, 22% MeOH and 20% DCM (v/v) in an acetonitrile mobile phase improved the liquid chromatographic separations and enhanced the resolution of chlorophyll *a* and zeaxanthin (**Figure D. 3.1**).

*D. 3.1.2. Analytical separation and identification of chlorophyll and carotenoids in field pea.* For chlorophyll and carotenoid quantification, extracts were separated on a reversed-phase (RP) $C_{30}$ column (250 mm x 4.6 mm, 3 μm particle size; Waters, YMC America, Newtown, PA, U.S.A.), preceded by a $C_{18}$ guard column (4.0 x 3.0 mm, SecurityGuard, Phenomenex, Torrance, CA, U.S.A.). Elution was controlled by Waters Alliance 2695 separations module using Empower 2 (bld 2154) software (Waters Corporation, Milford, MA U.S.A.). Isocratic elution (58:22:20, acetonitrile: methanol: dichloromethane) was used to separate compounds in the extracts, for up to 30 minutes at 1.0 mL $min^{-1}$. Compounds were detected by Waters 2998 photodiode array detector and data were collected over a wavelength range of 220 to 800 nm. Carotenoids and chlorophyll were quantified from peak area (absorbance units [AU] * sec) in chromatograms that were generated at 449 nm, the $\lambda_{max}$ for lutein under our mobile phase conditions (**Figure D. 3.1**). Flavonoid data were collected by photodiode array detector over a wavelength range of 198 to 500 nm. The retention time and UV-spectra of authenticated chemical reference compounds were used to identify the flavonoid, chlorophyll and carotenoid metabolites in field pea hulls.

*D. 3.1.3. Microscopy and histochemical staining.* Hull tissue was stained (*n*-butanol-HCl, 70:30, v/v) and scanned (Epson scanner, model 4490) to detect condensed tannin. In genotypes where a positive reaction occurred, the Butanol-HCl assay was used to quantify the CT from an average of three biological replicates.

**Table D. 3.2. Organic solvent series for extraction efficiency tests of chlorophyll, polar xanthophylls and carotenoids.**

| | ***Solubility (%)***[3] |
|---|---|

| *Solvent system*[1] | *Ratio (%)*[2] | β-carotene | Lutein | Chlorophyll |
|---|---|---|---|---|
| water/MeOH | *20/80* | 1.2 | 0.7 | 33.0 |
| Water/ethanol | *20/80* | 4.0 | 2.7 | 73.9 |
| water/acetone | *20/80* | 2.2 | 1.3 | 96.5 |
| MeOH | *100* | 4.6 | 26.4 | 81.4 |
| MeOH/DCM | *50/50* | 99.8 | 99.9 | 98.9 |
| DCM | *100* | 99.9 | 95.1 | 99.9 |
| DCM/chloroform | *40/60* | 100 | 84.1 | 97.2 |
| chloroform | *100* | 100 | 13.7 | 96.9 |
| hexane | *100* | 87.5 | 11.8 | 39.9 |

[1]in order of decreasing polarity;
[2]v/v, when two solvents were combined;
[3]calculated as ([solute dissolved] – [solute remaining]), relative to total starting weight for each chemical reference compound listed. Determination of undissolved solute weight: after a centrifugation step, the supernatant was removed and the dried residue was re-weighed, as described in the methods (D. 3.1.1).

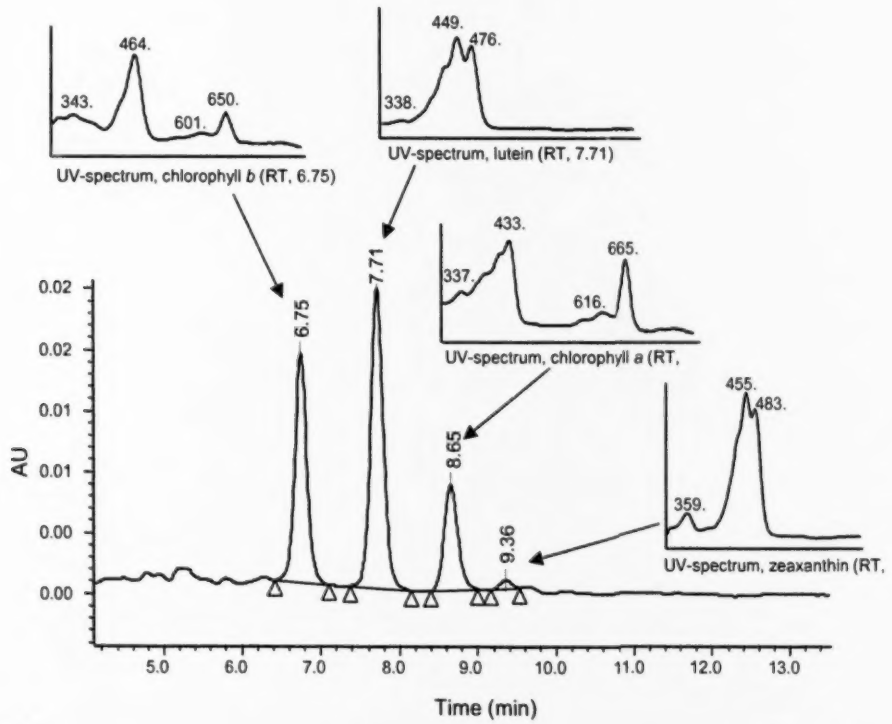



**Figure D. 3.1 Xanthophylls and chlorophylls in the mature hull tissue of CDC Striker.**
This elution profile was typical of UV-detectable, MeOH/DCM-soluble bioproducts in the mature hull tissue of field pea. The extracted components were identified by comparison to RT and UV-spectra of authentic reference compounds, separated under the same conditions. In mature tissue, violaxanthin and β-carotene were absent. Zeaxanthin was a trace component (9.36 min) and was not always detectable in the replicates of all genotypes.

*D. 3.1.2 Carotenoids and chlorophyll in developing field pea hulls.* A variety of polar xanthophylls, chlorophylls and carotenoids were detected in HPLC-separated extracts using UV-spectra to identify the components from developing hull tissue grown in the greenhouse. The three dominant bioproducts at all stages were the chlorophylls *a* and *b* and the polar xanthophyll, lutein (**Figure D. 3.2**). These compounds persisted throughout the growing stages and were present at maturity. Violaxanthin and β-carotene also accumulated in immature hull tissue, although these metabolites disappeared or were severely diminished over time, with the exception of β-carotene in the genotype Orb (**Figures D. 3.3, A, B**). Zeaxanthin and neoxanthin were evident in some of the immature stages of all three genotypes. However, the concentration was generally too low to quantify accurately.

Samples of Cooper, Cutlass and CDC Striker grown under field conditions were used to follow-up carotenoid production outdoors in comparison to developing seed grown in the greenhouse. Field samples were bulked due to low tissue yields and not all daf stages were available compared to greenhouse-grown material. The same compounds accumulated (violaxanthin, β-carotene, lutein, chlorophylls *a* and *b*) in field-grown seed as in greenhouse-grown material, although in lower concentrations. However, no zeaxanthin or neoxanthin were evident in field-grown developing tissue.

Although Cooper has less bleaching resistance than CDC Striker, there were no significant departures in the chlorophyll concentration or carotenoid bioproducts accumulated compared to CDC Striker grown in the field. When nearly mature Cooper seed (28 daf) was hand-decorticated, the underlying green cotyledon had bleached in patches, rather than uniformly losing the green colour. This observation suggests that the resistance-conferring mechanism occurred in some cells and not others. Evidence of this spatial distribution would be lost once the hull was uniformly ground for extraction.

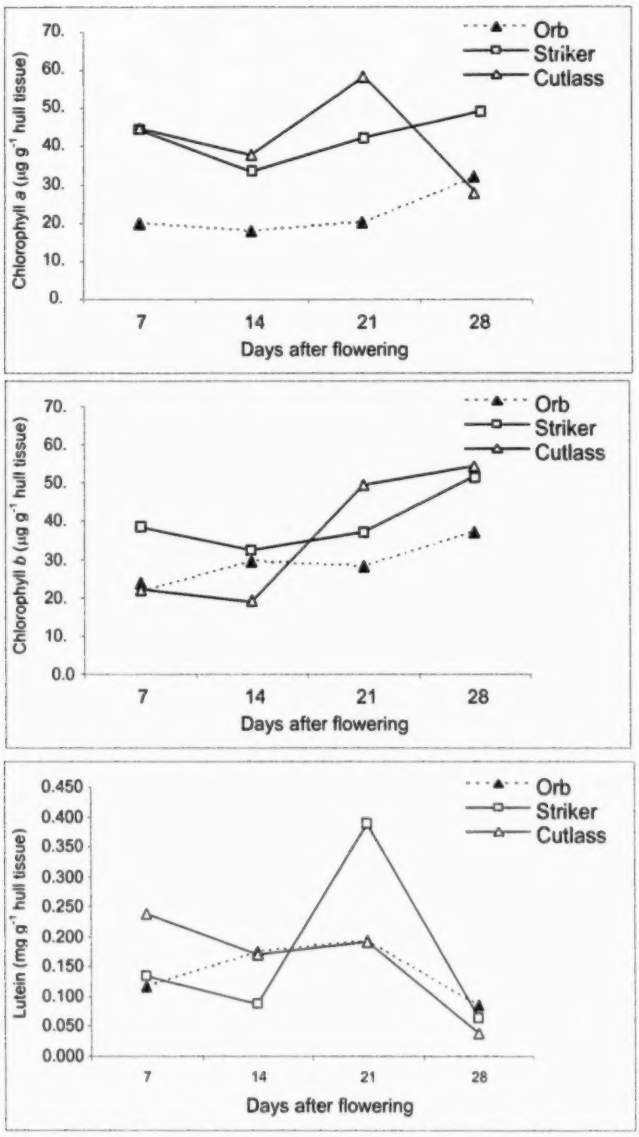
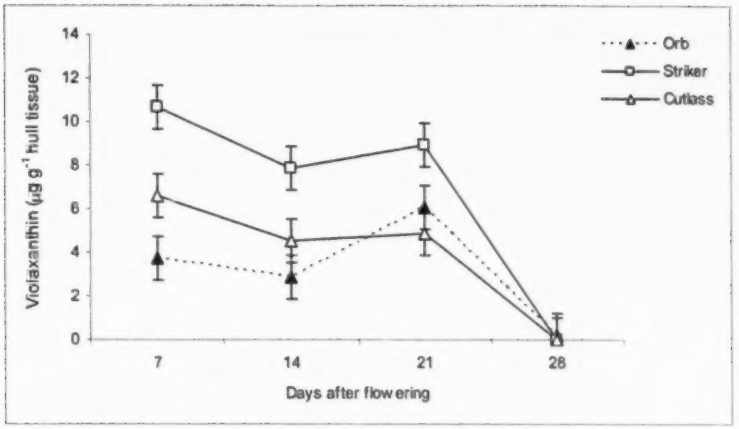



**Figure D. 3.2 Concentration of persistent carotenoid metabolites and chlorophylls in the developing hulls of field pea genotypes grown in a greenhouse environment.**
The compounds were detected by UV-photodiode array in HPLC separations. Data shown were derived from averaged values of three individual plants for each metabolite in each genotype at four sampling dates: 7, 14, 21 and 28 daf. These compounds persisted to maturity and were present after harvest of the dried seed (>35 daf).

**A.**




**B.**

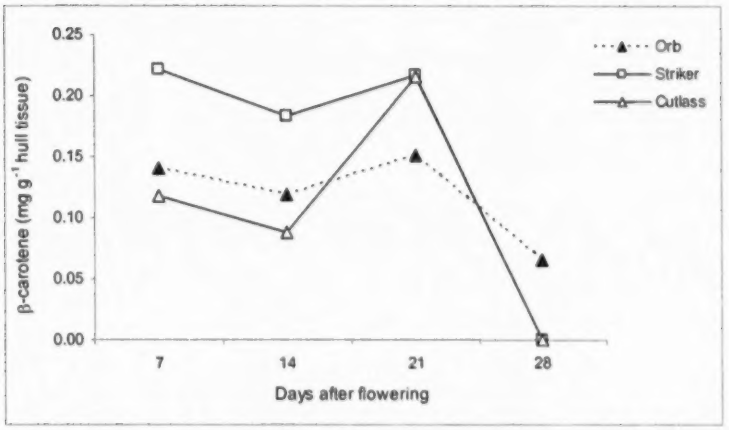
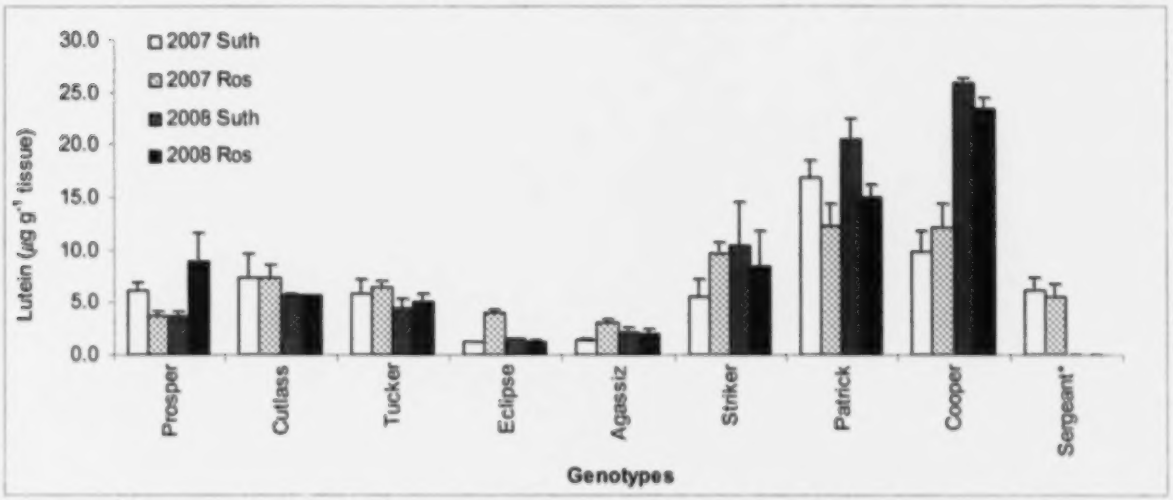



**Figure D. 3.3 Disappearance of two carotenoid metabolites during hull development of field pea.** Violaxanthin, β-carotene, neoxanthin and zeaxanthin were present in detectable amounts. Of these metabolites that disappeared during development, only violaxanthin (**A.**) and β-carotene (**B.**) were quantifiable in the immature hull.

*D. 3.1.3. Chemotype characterization of mature field pea hull phenotypes.* At harvest, the principal compounds that accumulated in the hulls in all genotypes were chlorophylls *a* and *b*, and a polar xanthophyll, lutein, at both locations and for both years (**Figures D. 3.4, 3.5**). The field pea genotypes with pigmented hull tissue (CDC Rocket and CDC Dundurn) contained higher concentrations of these compounds than any of the other genotypes. These trends prevailed over both years and both locations. In all analyses, genotype was significant in affecting the phenotypic accumulation of lutein and chlorophyll *b* over both year and location ($p = 0.016, 0.018$, respectively). This statistical difference was evident particularly for the pigmented types, especially in lutein accumulation which approached 128 µg $g^{-1}$ hull in CDC Dundurn (**Figure D. 3.4**). The G x E interaction was significant for chlorophyll *a* ($p =$

0.002), but was not significant for the other compounds ($p = 0.296$) taken over all genotypes. However, it was evident that two of the green cotyledon genotypes (CDC Patrick and Cooper) had the potential to accumulate lutein to a greater extent than the other green cotyledon varieties (**Figure D. 3.4**).



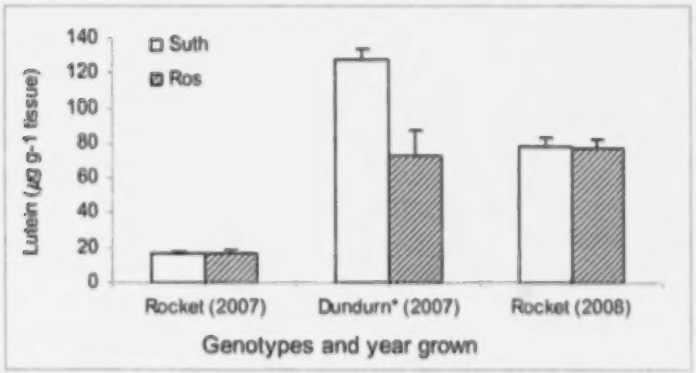




**Figure D. 3.4 Concentration of lutein in genotypes of mature hull tissue.**
Values represent the average of 3 biological replicates, harvested in 2007 and 2008 at two locations. The genotypes designated with '*' were not available in 2008. Lutein concentration in Maple and Dun types shown separately due to much higher values than in the yellow and green cotyledon types.

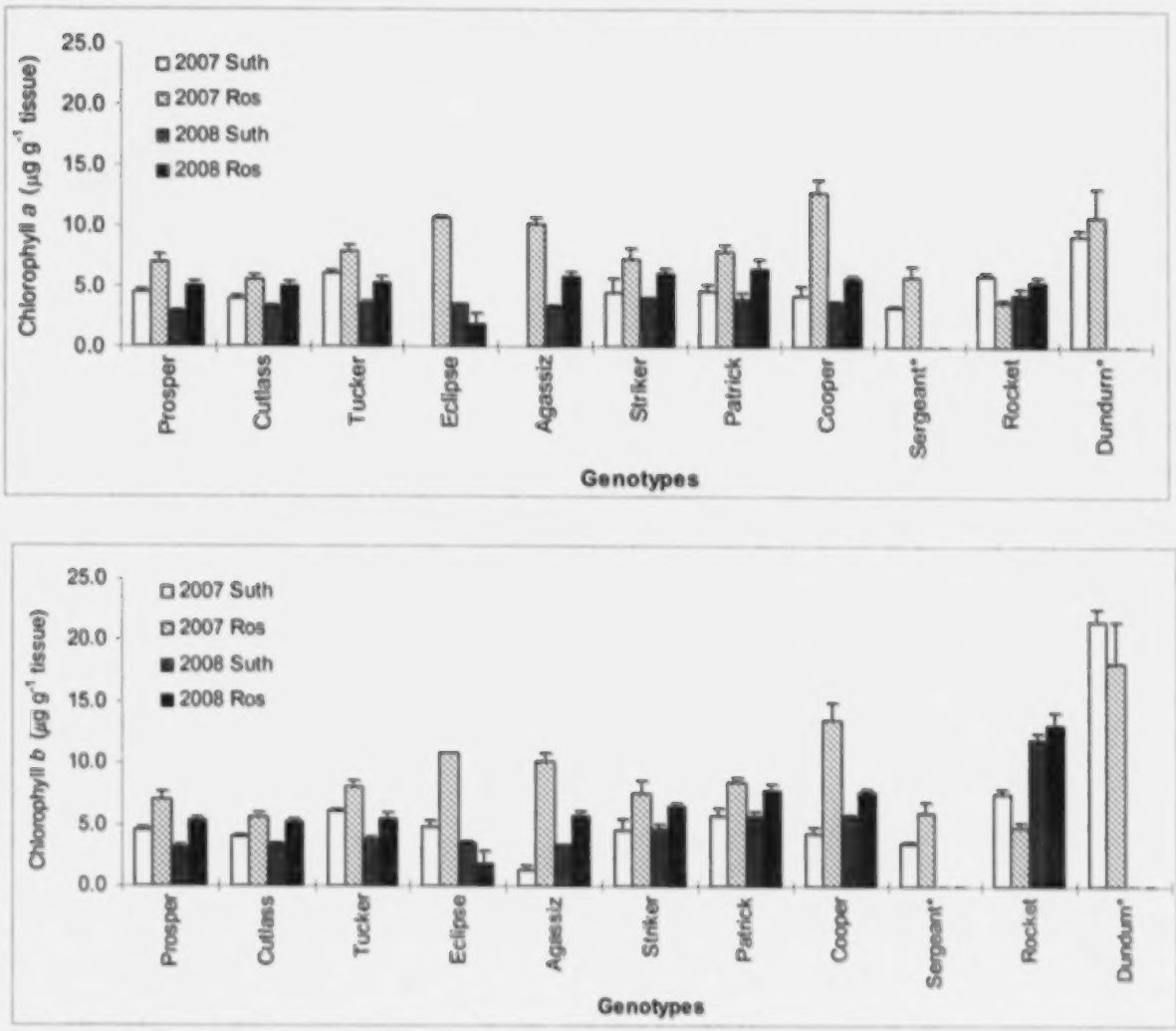



**Figure D. 3.5 Concentration of chlorophyll *a* and chlorophyll *b* in mature hull tissue.** Genotypes represent the maple and dun types, with pigmented hulls. Values represent the average of 3 biological replicates, harvested in 2007 and 2008 from two locations. The SE bars are not visible where variability was very low. Chlorophyll *a* was undetectable in Agassiz and Eclipse genotypes at Ros 2007. Genotypes marked with "*" were not available in 2008.

The classical evaluation of the *a/b* ratio (**Figure D. 3.6**) interprets the higher ratio as being typical of leaves in a well lit situation with no shading or abiotic stress (*71*). The *a/b* ratio decreases as shading or photo-induced damage increases, nutritional status decreases, or chloroplast senescence progresses. It has been predicted that the increase in chlorophyll *b* extends the wavelength range of photosynthetically active radiation that can be harvested by the photosynthetic light-harvesting apparatus in plants (*71*). Under this interpretation, lower abiotic or less physiological stress moves the ratio lower. Thus,

quantification of the *a/b* ratios (**Figure D. 3.6**) in genotypes of field pea indicated that yellow and green types may have endured more stress, perhaps due to the translucent seed coat, since CDC Dundurn and CDC Rocket, both with pigmented hulls, had lower *a/b* ratios. These latter genotypes have more complex metabolic profiles that included polyphenolics such as CT, which may be reflective of a photo-protectant effect of the CT content. In comparisons of wild type and transparent testa mutants (*tt*) of Arabidopsis, it was shown that CT afforded protection from physiological, abiotic and pathogenic stressors (*57, 72, 73*). The selection criteria during plant variety development may also have played a considerable role in the metabolic profiles of CDC Dundurn and CDC Rocket. Since the appearance due to the chlorophyll content would be masked by the CT colouring the hulls, uniformity of greenness would not be a noticeable phenotype and perhaps allows a greater variability to persist in the genotype.

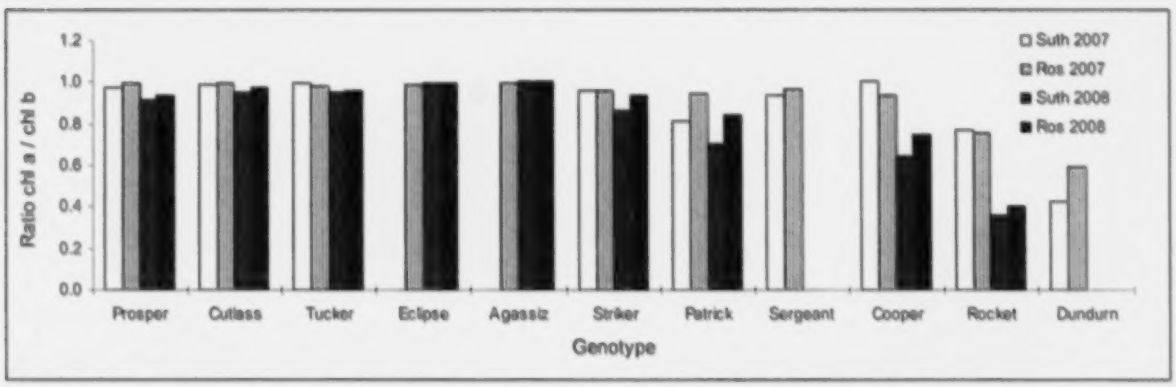



**Figure D. 3.6 The chlorophyll a/b ratio in hull tissue across genotypes of field pea.**
Ratio values are the equivalent of relative amounts of the two chlorophylls (if *a/b* = 1, the chlorophyll content is equivalent); values <1.00 mean that concentrations of chlorophyll *b* have increased relative to chlorophyll *a*. *Yellow cotyledon types*: CDC Prosper, Cutlass, CDC Tucker, Eclipse, Agassiz, CDC Rocket and CDC Dundurn. The latter two genotypes have pigmented hulls. *Green cotyledon types*: CDC Striker, CDC Patrick, Cooper, SW Sergeant. SW Sergeant and CDC Dundurn, not available in 2008.

*D. 3.1.4 Polyphenolics in mature field pea hulls.* In visually unpigmented hulls of field pea (pigment designations, **Table D. 3.1**), phenolics and flavonoids were not detected either by histochemical tests or from HPLC separation of extracts. Pigmentation differences were visually evident in CDC Rocket, with two subsets present (**Figure D. 3.7, A, B**). However, this appearance was not a different chemotype since the hulls of both subsets tested positive for CT (**Figure D. 3.7, C**). CDC Dundurn similarly reacted to butanol-HCl, indicating that CT was present in this tissue (**Figure D. 3.7, D**). The CT quantified by the butanol-HCl assay in the two pigmented hull genotypes had an average concentration

of 1.031 mg $g^{-1}$ hull tissue overall samples (n = 13, SE 0.106) with no significant difference between genotypes ($p$ = 0.875). Differential PPO activity may account for the differences in background colouration in these maple and dun types.

The unpigmented hulls did not contain any trace of CT in the HPLC separations of methanol and acetone extracts. The extracts from hulls of the maple and dun field peas contained detectable amounts of CT. However, it was evident that these observable amounts were not reflective of the actual concentration that was measured by the more aggressive heating in the butanol-HCl solvent reported above. In addition to CT, there were traces of UV-visible compounds in the methanol hull extracts that were equivalent in retention time and UV-spectra to di- and tri-hydroxybenzoates, comparable breakdown products to those reported elsewhere for flavonols (*26*). The origin of these hydroxybenzoates in field pea hull extracts was not elucidated.

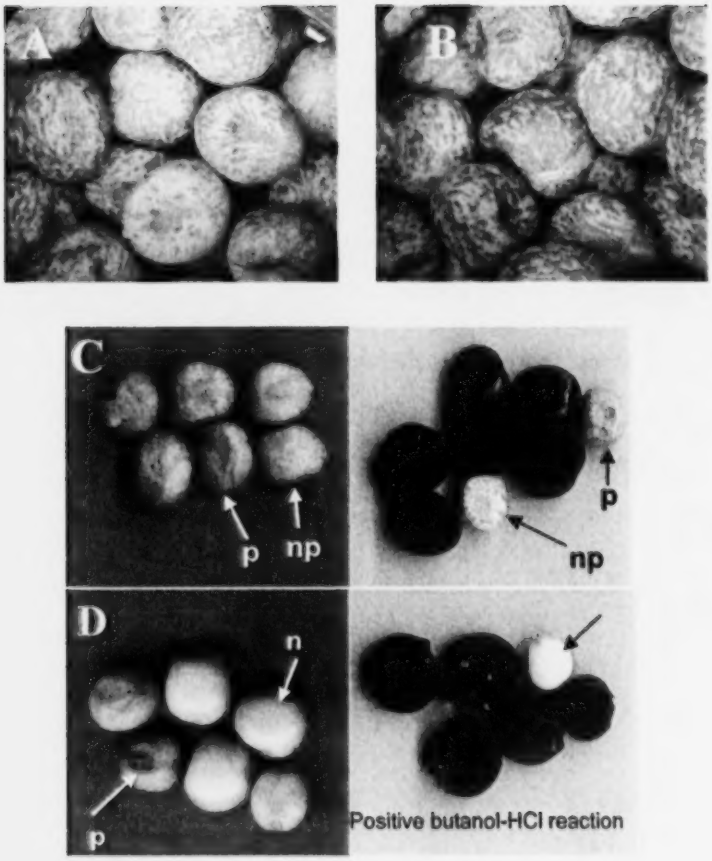



**Figure D. 3.7 Histochemical detection of condensed tannin in maple and dun genotypes.** The maple type, CDC Rocket (**A, B**) has a mix of background types, (**A**.) creamy and (**B**) reddish-brown. Both phenotypes have irregular brown patches, apparently in the outer integument, although the inner does not readily separate to confirm this. When the seeds are stained with butanol-HCl reagent (**C**), the hulls turn dark red and lose their apparent difference in colouring, confirmation that CT was uniformly accumulated. The dun type, CDC Dundurn (**D**) also has a mix of lighter and darker seed (white arrows). Similar to CDC Rocket, this genotype also contained CT uniformly in the hull. *Black arrows*, (**C, D**), un-imbibed seed for comparison; *np*, background not pigmented; *p*, pigmented background

### *D. 3.2* Biochemical evidence correlated with cotyledon bleaching resistance

Research in plant metabolism with respect to the mechanism of cotyledon bleaching may be furthered by this emerging portrayal of field pea hull tissue beyond the dietary aspect arising from biochemical documentation of field pea hulls. There is already strong experimental support that the hull

plays a pivotal role in preventing cotyledon bleaching in green types of field pea (*62*). When the hull was removed from CDC Striker, the cotyledons turned yellow after exposure to high intensity light. The green and yellow patchiness of the cotyledons discovered in Cooper may relate to sequestration of a bleaching resistant agent in the hull that similarly accumulated in a patchy manner. However, this sequestration was lost during the experiments reported here when the hull material from a seed sample was removed and ground as a composite tissue sample. The compound, carotenoid or otherwise that was responsible in preventing bleaching, would be discovered only if there was a significantly lower concentration overall of this salient biochemical.

There were no bioproduct data that unequivocally pointed to a specific carotenoid that correlated with lower levels of bleaching. There was, nevertheless, a trend towards lower chlorophyll *a* concentration in both NR green- and yellow-cotyledon genotypes compared to CDC Striker in both the greenhouse- and field-grown seed. In Orb, a green cotyledon, NR genotype grown in the greenhouse, the chlorophyll *a* profile displayed a similar tendency to lower accumulation as that shown by Cutlass and Cooper (**Figure D. 3.2**). The significance of these findings can be established only by monitoring R and NR genotypes over several environments and collecting analytical chlorophyll *a* values from each growth stage. It could well be that bleaching resistance is invoked early in the seed filling of field pea due to chlorophyll *a*, and these values at maturity may not be critical to the effect of chlorophyll *a* on bleaching resistance earlier in development.

### *D. 3.3 Future perspectives in field pea functional food attributes.*

By biochemically characterizing field pea hulls, a repository of functional food products has been documented in both developing and mature tissues. Analyses of developing hull revealed that this was a dynamic tissue containing a wide spectrum of precursors (β-carotene, violaxanthin, zeaxanthin) metabolically connected to important end products in plant metabolism such as lutein and ultimately, abscisic acid, an important molecule for regulating plant water status, seed dormancy and environmental response to cold stress (**Figure F. 2.2**) (*69, 73-75*).

Although a number of these products such as β-carotene, violaxanthin and neoxanthin disappeared during seed maturation, lutein persisted in concentrations in 1 g of hull (12 to 24 μg) that approached and in some cases (CDC Rocket, CDC Dundurn) exceeded, the RDA for carotenoids (**Figure D. 3.5**) (*40*). Although the dun and maple types were not used in the developing seed study, it is noteworthy

that a trace amount of β–carotene was detectable in all the mature samples from both Suth and Ros locations, even though the concentration was too low to quantify accurately. This discovery points toward the possible flexibility in selecting breeding lines during cultivar development that may lead to other field pea types having the nutritionally valuable β-carotene retained in the mature crop. This observation may be indicative that seed maturation can influence the occurrence of carotenoids and that somewhat immature seed could carry higher nutritional benefits even though appearance and processing characteristics may be compromised. The RDA for the provitamin A, β-carotene (1 μg retinol) (*40*) could be incentive to develop field pea cultivars that maintain the high β-carotene concentration to maturity that was detected at the 21-daf stage in developing seed of Cutlass and its persistence to 28 daf in Orb (**Figure D. 3.3, B**).

Higher lutein concentration was associated with the hulls of certain green cotyledon types. Lutein, a long-chain unsaturated hydrocarbon, represents a potentially valuable functional food component as an antioxidant in ameliorating macular degeneration (*14*). Whether the increased production of lutein in CDC Patrick and Cooper was a coincidence brought about by inbreeding due to crossing among green cotyledon types is unknown. It is equally possible that the higher lutein values coincided with other traits selected during plant variety development. Whatever the case, it was a serendipitous enhancement of a natural plant product with beneficial dietary advantages.

### D. 3.4 Field pea hulls as a value-added prospect in food supplements

In recent years, expansion of crop utilization strategies has become important in order to realize the best economic advantage for the complete crop, not only the traditional food source such as the cotyledons but also the so-called byproducts such as the hulls. Our results in chemotyping field pea hulls have highlighted a new perspective in this respect, because hulls represent a potentially valuable source of food-product enrichment due to the occurrence of essential dietary compounds. Since up to 10%, by weight, of the crop corresponds to the hull, an advantageous end-use for this byproduct could represent an important value-added trait for field pea as a total carotenoid source (**Figure D. 3.8**).

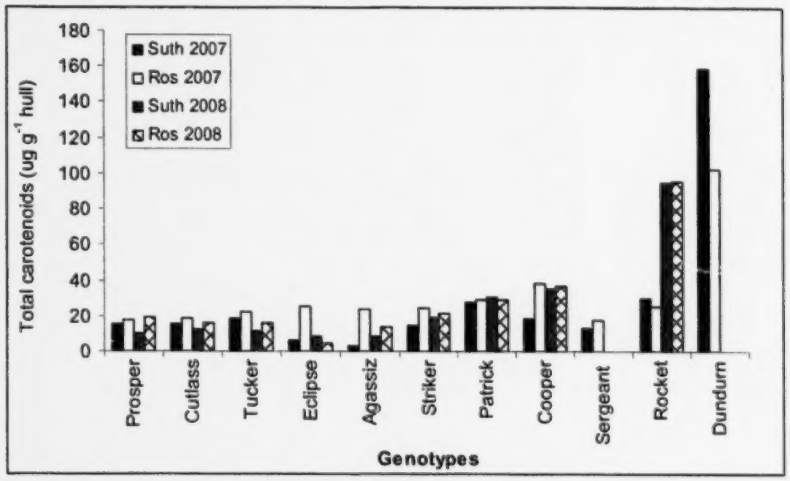



**Figure D. 3.8 Comparison of total carotenoid accumulation in mature field pea hulls.**

Chlorophylls *a* and *b*, and lutein were combined to give a relative concentration (μg $g^{-1}$ hull) across all genotypes. *Yellow cotyledon types*: CDC Prosper, Cutlass, CDC Tucker, Eclipse, Agassiz, CDC Rocket and CDC Dundurn. The latter two genotypes have pigmented hulls. *Green cotyledon types*: CDC Striker, CDC Patrick, Cooper, SW Sergeant. SW Sergeant and CDC Dundurn, not available in 2008.

Since consumption of hulls alone is likely unappealing, ground field pea hulls could be incorporated without any refinement as a food supplement into many processed foods such as minced meat products or baked goods. Sensory panel evaluation of cooked meat products that incorporated up to 12% lentil flour were positively influenced by the modified products' juiciness and tenderness compared to unadulterated meat patties (*76*), which suggests that field pea hull flour could also be used successfully. Ground flour made from field pea hulls has been evaluated in baked goods but not as a modifier of meat products (*77*). However, in a different investigation, the field pea hull produced very fine flour that fortified familiar snack food products and resulted in high patient acceptance (*78*). Clinical studies of the dietary aspect of including field pea hull fibre in these snack foods for elderly patients demonstrated an advantage to their health due to increased elimination function (*78*). Health improvements may have also been reflective of an enriched functional food due to the unrecognized contribution of the accumulated carotenoid and antioxidative chlorophyll bioproducts in the hulls.

Consumption of whole field pea, in soups or curries for instance, may carry advantages as a healthier alternative to processed food products and plant-based extracts, with the hull attributes playing a decisive roll as an unadulterated natural source of total carotenoids. Since the RDA of provitamin A

(derived from β-carotene, **Figure F. 2.2**), is in the 1 μg range, consumption of 10 g field pea could fulfill this dietary recommendation. Moreover, fibre would be included naturally in this type of diet and contribute to balancing the other components in the meal. The addition of high fibre to diets without the balance of other dietary components has previously resulted in insignificant or unsubstantiated health gains (*1*). There is a growing body of evidence that supports whole foods consumption as a desirable route to inexpensive, financially approachable nutrition to benefit health in a functional food context (*1, 4, 7, 8, 79, 80*).

### *D. 4 Faba Bean*

As a potentially re-emerging Saskatchewan crop, faba bean is poised to be either consumed whole, or fractionated, leaving the seed coat as a potential source of nutritional supplements. The biochemical potential of the hulls was unknown previously. Currently, the faba bean lines under development have not been used for evaluation due to the early stages of the cultivar development program. A brief overview of CT in faba was undertaken here of parental material sourced from other countries (**Table D. 4.1**). Different cultivars have been examined by other researchers and the presence of a number of polyphenolic products documented in these (*81, 82*).

**Table D. 4.1. Faba bean (*Vicia faba*) genotypes**

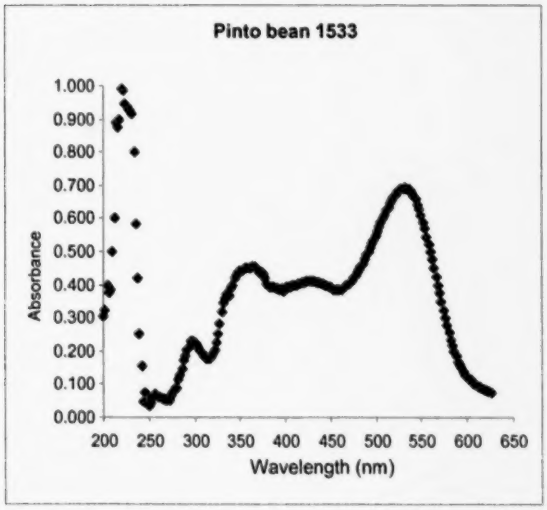
| *Genotypes* | *Harvest date* | *Plot #* | *Location* | *Seed coat type* |
|---|---|---|---|---|
| AZ10<br>'zero' tannin type | 2007 | 4609<br>4625<br>4648 | Outlook | light tan;<br>tan hilum |
| Disco<br>'zero' tannin type | 2007 | 4605<br>4618<br>4641 | Outlook | light tan;<br>tan hilium |
| Dixie<br>'zero' tannin type | 2007 | 4616<br>4628<br>4647 | Outlook | light tan;<br>tan hilum |
| CDC Fatima | 2007 | 4607<br>4626<br>4645 | Outlook | light tan;<br>black hilum |
| Gloria<br>'zero' tannin type | 2007 | 4614<br>4629<br>4642 | Outlook | light tan;<br>black hilum |
| Snowbird<br>'zero' tannin type | 2007 | 4610<br>4619<br>4644 | Outlook | very light tan;<br>tan hilum |
| SSNS-1 | 2007 | 4615<br>4631<br>4636 | Outlook | light tan;<br>black hilum |

### *D. 4.1 Overview of key results from faba bean investigations*

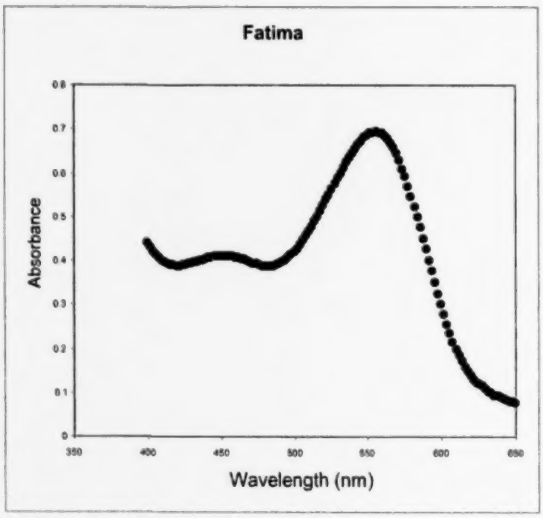
Of the genotypes available here, only CDC Fatima and SSNS-1 had CT in the seed coat. The dark tan colour of the other material produced a negative brown reaction in the butanol-HCl assay. The faba bean wavelength scans (**Figure D. 4.1**; 200 to 850 nm) are from extracts of faba bean seed coat tissue (graphs 2 to 8) (graph 1 is control CT from pinto bean). Typical BuOH-HCl wavelength scans were obtained for **samples 1 to 3**. The post-assay preparations were deep burgundy red and required 50x dilution for measuring the absorbance. Scan 1 (pinto bean) had a maximal absorbance at 538 nm and the "high tannin" faba bean genotypes absorbed maximally at 556 nm. Non-typical scans were

obtained for samples 5 to 8. The non-typical scans appeared to separate into one of two categories. **Samples 4** and **5** (Figure D. 4.1) produced very dark brown extracts that were diluted 1:10 and 1:5 for measuring the absorbance. The diluted solutions were a pinky-brown and did not display any associated maximum other than at 280 nm (possibly related to protein and present in all the samples). **Samples 6 to 8** were not diluted, and produced a lighter 'tea-brown' solution that absorbed around 450 nm. Overall, it may be that the genotypes of faba used for profiling had converted available CT to quinones which do not react with the reagent to produce the diagnostic dark red reaction. While there are two different genes associated with the 'zero tannin' phenotype: *zt1* and *zt2*, they did not appear to correlate with the two different results.

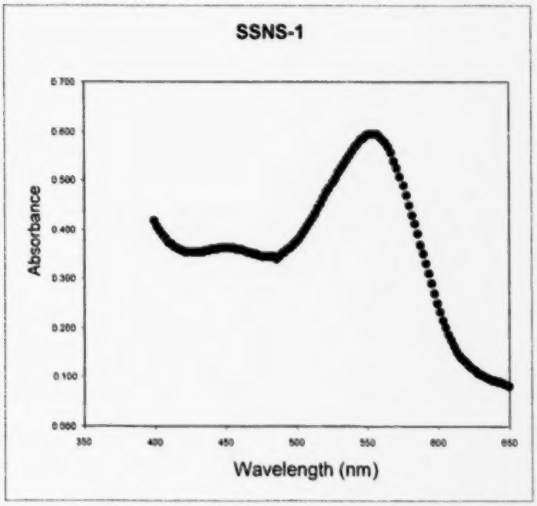
**1**



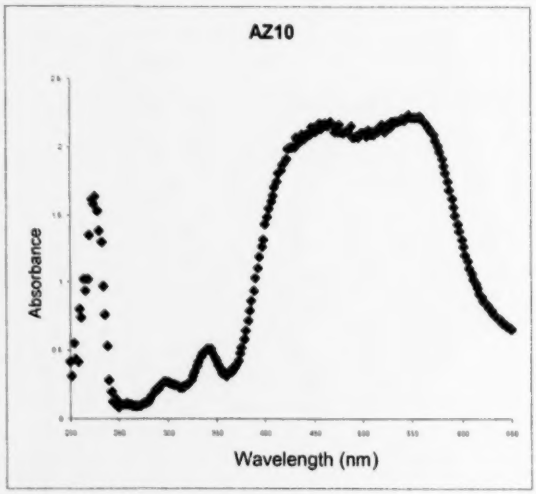

**2**




**3**




**4**



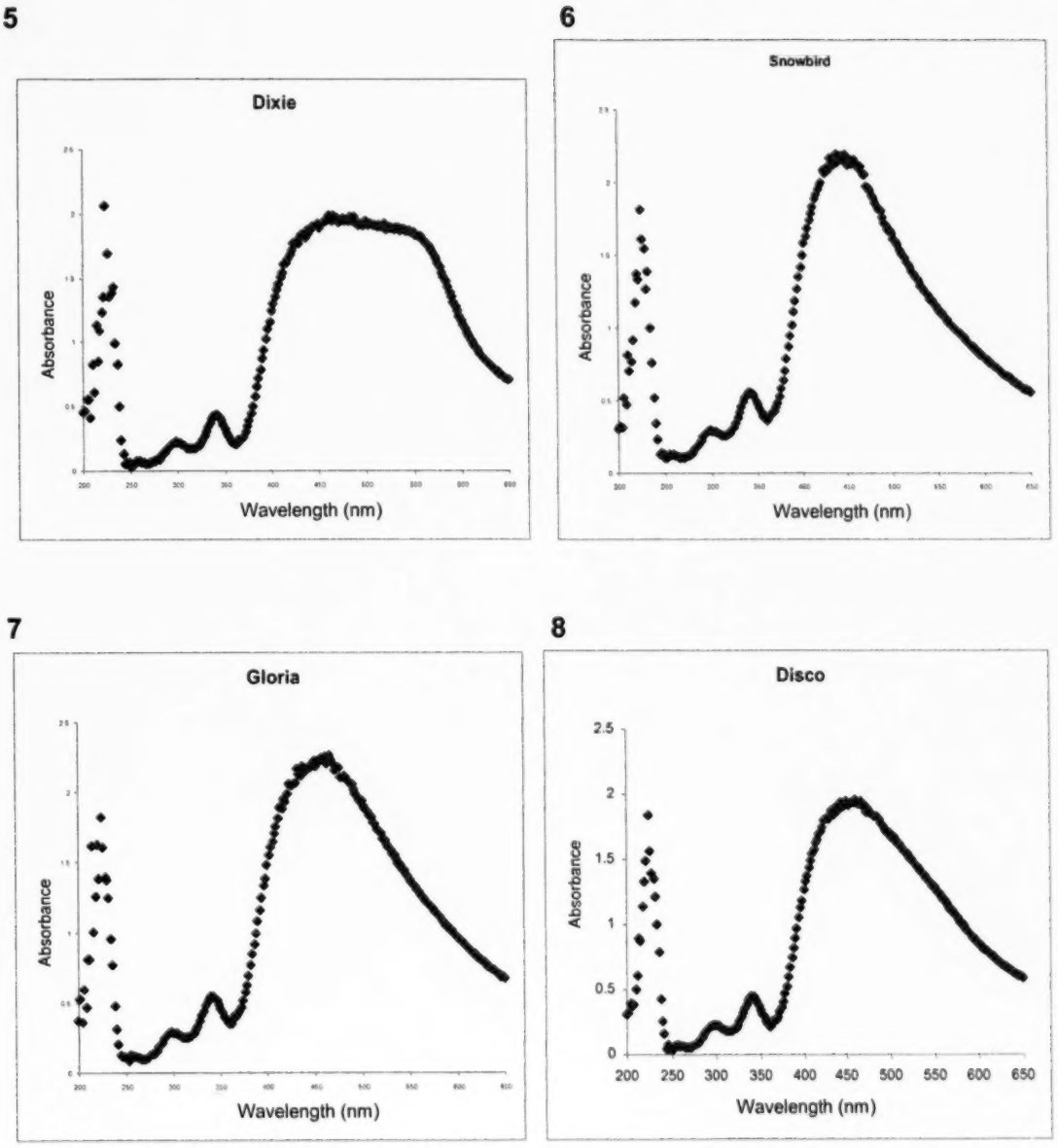



**Figure D. 4.1 Butanol-HCl wavelength scans of faba bean genotypes.**
The wavelength scans are from extracts of faba bean seed coat tissue (**graphs 2 to 8**) (**graph 1**, control assay, semi-purified CT from pinto bean). Extracts were prepared using the butanol-HCl assay . The genotype codes are detailed in **Table D. 4.1.**

### *D. 5 Additional research results*

*D. 5.1 Field Pea Seed Residue: a Potential Alternative Weed Control Agent.* Field pea seed from bin cleaning operations stored overwinter on nearby cropland was observed to correlate with weed and crop growth suppression for up to three subsequent years. To explore the phenomenon more explicitly, plant growth suppression trials were undertaken with soil sampled 18 mo apart from two locations which had contained field pea seed residues. Test plant species grown in the residue-affected and nearby residue-free soils were compared in greenhouse experiments. Germination was either fully inhibited or emergence was delayed by more than one week. Dry matter accumulation of test species grown in residue-affected soil was significantly reduced compared to dry matter of these test species grown in residue-free soil ($P < 0.0001$). Canola and field pea were inhibited more than wheat and green foxtail over both years. Greenhouse trials also revealed that germination of wild oat was inhibited in the residue-affected soils, although overall, wheat and grassy weeds were less suppressed than dicots. Significant reductions of weed species diversity and abundance were correlated to residue-affected soils ($P < 0.0001$) when compared to residue-free soils using multi-response permutations procedures. Germination of wheat and canola seed was inhibited, using aqueous extracts of weathered pea seeds or extracts of the residue-affected soil in bioassays in sterile media. An allelopathic response was proposed to explain the above results, indicating a need for further research on this system. Weed management strategies could be developed with field pea seed residues that provide innovative weed control techniques. (Published, Weed Science, 2010) (*83*).

*D. 5.2 Non-darkening pinto and cranberry bean germplasm.* Breeding lines under development at other institutions have non-darkening rather than slow-darkening phenotypes. Initial histochemistry and preliminary HPLC separations indicate that compared to the slow darkening parental line, 1533-15, the non-darkening cranberry cv. Wit-rood, and non-darkening pinto line KVxUI-1 do not appear to accumulate CT. Since we have hypothesized that flavonols are instigators, if not solely responsible, for post-harvest colour change in the seed coat, it was of considerable interest to find that progeny contained both kaempferol and quercetin as well as other unidentified flavonols **(Figure D. 5.2)**. The gene controlling the non-darkening phenotype is distinct from the one that controls slow-darkening (Eldadr and Bett, manuscript in prep.). It may be that a regulatory element has down-regulated PPO so that although the substrate was present, little or no enzyme activity occurred. This is preliminary research and will not be further pursued until appropriate RIL populations have been developed. We are not pursuing the non-darkening phenotype for commercial bean cultivars because the gene controlling this phenotype also appears to cause the pattern on the seed coat to appear washed-out. In

addition, development of a non-darkening bean might lead to the temptation to store beans too long before selling which leads to the hard-to-cook phenomenon.

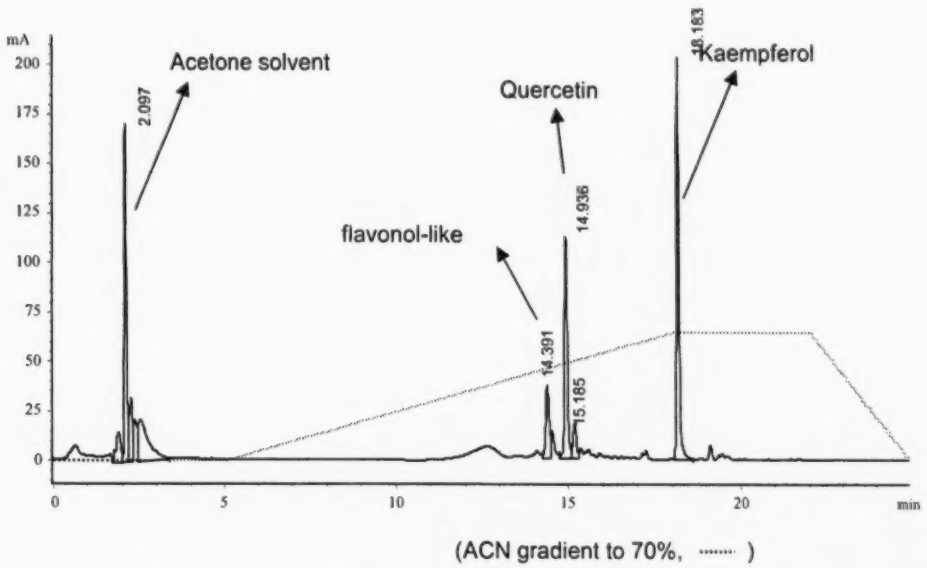



**Figure D. 5.2 UV-spectral analysis of KVxUI-1, a non-darkening pinto bean line.**
Reversed-phase, $C_{18}$ separation of a seed coat methanol extract was achieved in an acetonitrile gradient from 5% to 80 %. UV-photodiode array detection was used to identify flavonols in the chromatogram monitored at 365 nm. In this extract, there were no condensed tannins.

## E. CONCLUSIONS

This project enhanced our knowledge of naturally-occurring health products in pulse crops adapted for Saskatchewan. Biochemical tools were developed for improvement of pulse crops with a particular emphasis on the value of the seed coat (hulls), an under-utilized plant product. Development of pulses with novel dietary attributes can complement the marketing strategies currently used to promote dry bean and field pea consumption.

The seed coat was of particular importance in this research because we determined that up to 10% of the weight of the crop consists of this tissue. Hence, biochemical profiling data added to our knowledge of the seed coat in ways that could generate added-value and increase the overall marketability beyond preference for an attractive seed colour and pattern in dry bean as well as promoting consumption of unsplit field pea. Quality of the dry bean crop, like that for most pulses, is based primarily on visual characteristics of seeds such as colour, size and shape. We determined that

understanding seed coat colour in dry bean benefited from chemotyping and resulted in an effective screening technique that could be complementary to use of molecular markers.

This is important knowledge with the potential for an economic advantage for Saskatchewan growers. The accumulation of a range of health-giving bioproducts, such as flavonoids and carotenoids, in the pulse seed coat provides a splendid potential for an unrealized source of dietary components. These classes of compounds have been reported as important dietary factors using *in vitro* techniques, but follow-up clinical studies are now required to make this a reality. To develop healthful pulse products, it is critical to understand the nature of the pigment in the seed coat, and what other health-giving bioproducts occur as colourless components. Very little is known about the accumulation of these added-value dietary prospects. The emerging biochemical profiles of beans and peas could be the means of developing a reputation for Saskatchewan as a promising supplier of highly flexible pulse crops with regard to different utilization strategies.

# F. APPENDICES

## F. 1 PRESENTATIONS AND PUBLICATIONS:

Marles, M.A.S., Warkentin, T.D., and Holm, F.A. (2010) Field pea seed residue: a potential alternative weed control agent. *Weed Science*. 58, 433-441. (DOI: 10.1614/WS-D-10-00015.1).

Marles M.A.S, Balasubramanian, P. and Bett K.E. (2010). Differential Accumulation of Polyphenolics in Black Bean Genotypes Grown in Four Environments. *Journal of Agricultural and Food Chemistry*, 58, 7001–7006. (DOI:10.1021/jf100630g).

Marles M.A.S, Coulman B.E. and Bett K.E. (2008). Interference of condensed tannin in fibre and lignin analyses of dry bean and forage crops. *Journal of Agricultural and Food Chemistry* 56 (21): 9797–9802. doi: 10.1021/jf800888r.

Marles M. A. S., Warkentin T.D and Holm F.A. (2010). Field pea mulches: The potential for low-cost weed control. Pulse Days, January 11-12, Saskatoon, SK. p. 36. Also presented at 2010 Soils & Crops Workshop, February 25th, 2010, Saskatoon, SK.

Elsadr H., Marles M. A. S, Caldas G., Blair M. W. and Bett K. E (2009). Phenotypic characterization of condensed tannin accumulation in five dry bean genotypes. pp. 20- 22. Bean Improvement Cooperative. Nov. 2-5. Fort Collins, WY.

Marles M. A. S., Holm F.A., and Warkentin T.D. (2008). Allelopathic attributes of field pea. 7th Canadian Pulse Research Workshop. Nov. 4-7. Winnipeg, MB, Canada. pp. 108-109.

Marles M. A. S., Balasubramanian, P., and Bett K.E. (2008). Metabolic endpoints in legume seed coat pigmentation pathways. Banff Conference on Plant Metabolism. July 30 to Aug. 3. Banff Centre, Banff, AB, Canada. p. 21.

## F. 2 INFORMATION OF BENEFIT TO PLANT BREEDERS, PRODUCERS AND PROCESSORS: *a catalogue of cultivars and associated chemotypes.* (To be appended when the red bean profiling is completed).

### F. 2.1 METABOLIC PATHWAY SCHEMATICS

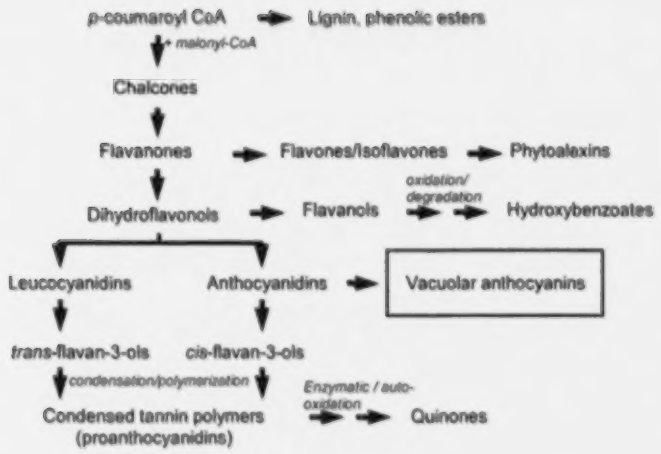



**Figure F. 2.1 Flavonoid metabolism.** Key flavonoids such as flavanones, flavonols and condensed tannin are shown as dynamic products that can contribute to other biosynthetic cascades. The coumarates and chalcones are precursors to flavonoids and possess bioactivities in plant defense as well as building blocks for complex polymers such as lignin.

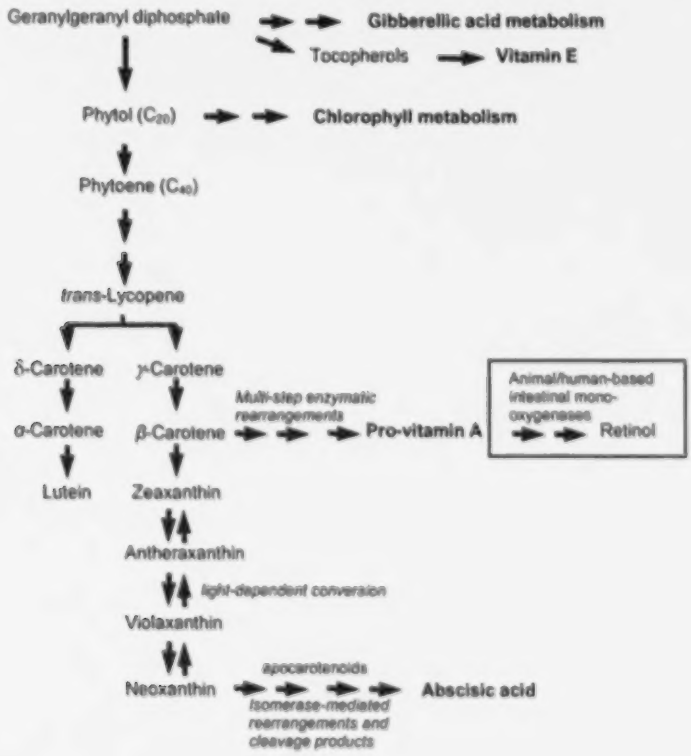



**Figure F. 2.2 Carotenoid metabolism.** Terpenoid biosynthesis produces geranylgeranyl diphosphate, a precursor for subsequent biosynthesis of other natural plant products, including essential vitamins, provitamins, chlorophyll and plant growth hormones.

## F. 3 PERSONNEL:

Dr. Susan Marles – lead researcher
Field, lab and greenhouse technical support.

## F. 4 EQUIPMENT PURCHASED OR RENTED:

Lab and field equipment rental.
Growth facilities rental.
No major equipment purchased for this project.

## F. 5 PROJECT DEVELOPED MATERIALS

The catalogue of cultivars and associated chemotypes will be available on our Pulse Crop Research website (www.pulse.usask.ca) this spring.

## F. 6 PROJECT PHOTOS AND FIGURES

Some photos of various aspects of this work are available on request. Several figures are unavailable, however, because the copyright is held by the journal in which the research was published.

## F. 7 ACKNOWLEDGEMENTS:

The Saskatchewan Agricultural Development Fund has been and will be acknowledged in all presentations and journal articles where this research project is mentioned.

The black and red bean genotypes from multiple location yield trials were graciously supplied by Dr. P. Balasubramanian, dry bean breeder at AAFC Lethbridge.

The assistance of the CDC Pulse Crop Research Field Crew in providing field pea samples is gratefully acknowledged.

## F. 8 EXPENSE STATEMENT:

Will be forwarded.

## F. 9 LIST OF ABBREVIATIONS (9.1) AND DETAILS OF STATISTICAL PROCEDURES (9.2)

### 9.1 *Abbreviations*

condensed tannins, CT; genotype by environment, G x E; polyphenol oxidase, PPO; recommended daily amount, RDA; ultra-violet, UV.

### 9.2 *Statistical procedures*

Results were converted to μg or mg $g^{-1}$ seed coat, as appropriate; error bars represent standard error of the means (SE) in the graphs. For each bioproduct quantified, individual values are reported as a mean of three biological replicates for every genotype (technical replicates were averaged to produce an average of each biological replicate). Data were analysed using SAS PROC Mixed (SAS 9.2 for Windows V5.1.2600, SAS Institute, Cary, NC) to determine whether there were significant differences among genotypes with respect to specific accumulated bioproducts, a significant replicate effect and to test whether there was an interaction with the location or the year in which the plants were grown (G x E effect).

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